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(54) **Fatty acid and derivatives thereof in treatment or prophylaxis of thrombo-embolic conditions**

(57) (All–Z–)–5,8,11,14,17-eicosapentaenoic acid or a derivative thereof for administration to a mammal, including man, for treatment or prophylaxis of thrombo-embolic conditions, for increasing bleeding time, for disintegrating or dispersing already formed thrombi or platelet clumps for modifying or controlling adherence of blood platelets to damaged tissue, for rendering blood platelets less readily aggregatable and/or more readily disaggregatable from one another, and for adding to blood in extra-corporeal circulation.

Pharmaceutical formulations contain (all–Z)–5,8,11,14,17-eicosapentaenoic acid or a pharmaceutically acceptable salt, ester or amide thereof.

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SPECIFICATION

Fatty acid and derivatives thereof in treatment or prophylaxis of thrombo-embolic conditions

The present invention relates to the treatment or prophylaxis of thrombo-embolic conditions.

Although it is known that many substances can affect platelet aggregation, it cannot be predicted from a knowledge of the effect of a particular substance on aggregation of platelets *in vitro*, whether or not the substance will have an inhibitory or stimulatory (or neutral) effect on thrombus formation *in vivo*. This is largely because it is not known what initiates formation of a thrombus or embolus in, for example, a stroke or myocardial infarction. As an example of this unpredictability, aspirin is a good inhibitor of platelet aggregation *in vitro* and *in vivo*, but it is not an anti-thrombotic agent, in particular it cannot disperse a preformed thrombus.

M. J. Silver, J. B. Smith, *et al.*, (Prostaglandins Dec. 1973, Vol.4, No.6, pages 863 to 875) showed that many compounds can influence *in vitro* the platelet aggregating effects produced by the essential dietary component arachidonic acid (5,8,11,14-eicosatetraenoic acid, alternatively C20:4; n–6 acid, i.e. a fatty acid containing 20 carbon atoms having 4 carbon-to-carbon *cis*-double bonds, the one at the highest numbered position being at a position 6 bonds from the end of the molecule remote from the carboxyl group, and n being the number of carbon atoms in the straight chain). These *in vitro* tests in human citrated platelet rich plasma cannot be unambiguously related to *in vivo* behaviour in the thrombus formation-prone mammal, including man. M. J. Silver *et al.* found in their tests that the platelet aggregation induced by arachidonic acid, as sodium arachidonate, can be inhibited by many materials including adenosine; β-naphthol; non-steroidal, anti-inflammatory agents such as indomethacin, sodium salicylate and aspirin; human albumin; unsaturated fatty acids, such as 11,14,17-eicosatrienoic acid, 8,11,14-eicosatrienoic acid (dihomo-γ-linolenic acid, DHLA), 5,8,11,14,17-eicosapentaenoic acid, 5,8,11,14-eicosatetraynoic acid, and 4,7,10,13,16,19-docosahexaenoic acid. They also found that the platelet aggregation induced by collagen and a second wave of platelet aggregation induced by adenosine diphosphate (ADP) could be inhibited by β-naphthol, aspirin, 8,11,14-eicosatrienoic acid, 5,8,11,14,17-eicosapentaenoic acid and human albumin. Silver *et al.* further found that various fatty acids on their own did not induce platelet aggregation. The acids they mentioned were 8,11,14-eicosatrienoic acid; 11,14,17-eicosatrienoic acid; 5,8,11,14,17-eicosapentaenoic acid, 5,8,11,14-eicosatetraynoic acid; 4,7,10,13,16,19-docosahexaenoic acid; linolenic acid; linoleic acid; oleic acid; arachidic acid; stearic acid; and decanoic acid.

It will be appreciated that many of the compounds found by Silver *et al.* to be anti-aggregatory are unsuitable for use in therapy. For example, adenosine is rapidly absorbed by cells and so would not be available in the body for long enough to be of value. β-naphthol is toxic as it is a phenolic compound. Albumin is unsuitable because it would put an undesirable load on the kidney and give rise to glomerulant damage in the kidney. As non-steroidal anti-inflammatory agents frequently give rise to gastric lesions, they should preferably be avoided in any therapy requiring long term prophylactic oral administration as is often desirable in cardiovascular treatments.

Silver *et al.* appear to conclude that arachidonic acid has an important place in hemostasis and thrombosis, and that its effects can be inhibited *in vitro* by various compounds, particularly albumin. They suggested that albumin may be an important controlling factor in hemostasis and that the ability of albumin to bind arachidonic acid in circulating blood might be the way it inhibits the effects of arachidonic acid. They further suggested that the net binding capacity of albumin for arachidonic acid may depend on, for example, the availability of binding sites and competition between arachidonic acid, other fatty acids and other classes of substances for those sites. Presumably, therefore, the more competing substances there are available, particularly other fatty acids, the more free arachidonic acid there would be and the more likely platelet aggregation would be, and therefore if these phenomena were to be related, the more likely thrombus formation would be. This suggests that other fatty acids should be removed from the diet.

Attempts have been made to investigate in man the effects of various fatty acids on diseases involving thrombus formation, but no clear conclusion has emerged.

For example, the Norwegian Vegetable Oil Experiment of 1956–66 was carried out before the work of Silver *et al* and was reported by H. Natvig, Chr. F. Borchgrevink, *et al* in Scand. J. Clin. Lab. Invest. *22*, Suppl. 105, 1–20, (1968). The study compared the effects on human mortality rates caused by various coronary heart diseases, including myocardial infarction, of two diets, one containing sunflower seed oil (about 63% of linoleic acid) and the other containing linseed oil (about 55% of linolenic acid); 10 ml. of either oil being taken per day. The group taking the more highly unsaturated linolenic acid was found to be *more* at risk than the group taking the linoleic acid.

Linoleic acid, and, in rats, eicosapentaenoic and docosahexaenoic acids are known to decrease

blood plasma cholesterol levels, which are believed to be connected with atherosclerosis. Atherosclerosis is often found in persons who have suffered from a myocardial infarct. However, there appears to be no causal relationship, because Robertson (Lancet, (1959), i, 44) found that in Jamaica, although extensive atherosclerosis is regularly found in the native population at necropsy, it is very seldom associated with secondary thrombi or with myocardial infarction. Further, myocardial infarcts can occur in the absence of highly developed atherosclerosis.

Yet another possible dietary factor that has been suggested (P.B. Kernoff, A.L. Willis, K.J. Stone, J.A. Davis and G.P. McNicol, British Med. J., 1977, *2*, 1441–1444) as helping to inhibit thrombosis is DHLA. DHLA is a biosynthetic precursor of prostaglandin E_1 (PGE_1), which is a powerful inhibitor of platelet function, and was said to be attractive as an anti-thrombotic agent. It was found that there was, as hoped, a rise (mean 55%) in production of the desirable PGE_1 but in six men out of the eight tested there was also a rise (mean 33%) in production of the undesirable prostaglandin E_2 (PGE_2). Furthermore these results were not clearly dose related. There was also a lowering of heparin-neutralising activity of plasma, and this activity has been found to be high in thrombotic states. However, the authors did not know the extent to which heparin-neutralising activity reflects basic pathological mechanisms, and so its relationship with thrombosis was unclear.

The authors of the paper speculated that "Perhaps small doses of DHLA may be equally if not more effective than major dietary manipulations in preventing and treating these conditions" i.e. atherosclerosis and coronary heart disease. However, the author of an editorial in the same edition of the Journal (pages 1437 and 1438) was more cautious and thought that "Trials of agents and regimens that modify the platelet prostaglandin mechanisms must be carried out before we can tell whether the results obtained by McNicol and his colleagues have any clinical application". The reasons for his caution lay in the ignorance that exists of the mechanisms involved *in vivo* in thrombotic situations, when investigative tests have only been carried out on shed blood.

This at least partially attractive work with DHLA throws some doubt on the frequently quoted view that unsaturated fatty acids in the diet are more beneficial than their more saturated analogues, especially as the even less saturated linoleic and linolenic acids can be metabolised to DHLA. This doubt is strengthened by the fact that arachidonic acid which is undesirable (see Silver *et al* and Kernoff *et al* above) is even more unsaturated (four carbon-carbon *cis*-double bonds) than DHLA (three *cis*-carbon-carbon double bonds).

We have now surprisingly found that among the many fatty acids (all Z)-5,8,11,14,17-eicosapentaenoic acid or its salts, esters or amides can be used to treat effectively, or provide effective prophylaxis against, thrombo-embolic conditions, hereinafter referred to simply as thrombosis. Examples of conditions where our findings may be useful are in the treatment or prophylaxis of cardiovascular disease mediated by the formation of a thrombus or thrombi, for example myocardial infarction, stroke, or deep vein thrombosis during surgical operations.

We have found that (all Z)-5,8,11,14,17-eicosapentaenoic acid (hereinafter referred to simply as eicosapentaenoic acid, also known as eicosapentaenoic acid) when injected intravenously into rabbits increases their bleeding time, thus demonstrating a decrease in the tendency of the blood to produce thrombi or adhere to damaged tissue, thus enabling one to modify and/or control wound-healing. When infused into rabbit lung, eicosapentaenoic acid gives rise to a substance which has a powerful anti-aggregatory action on blood platelets.

Eicosapentaenoic acid also has the unusual and important ability to disperse or disintegrate already formed thrombi or platelet clumps. For example, blood from an anaesthetised rabbit was allowed to drip over a continuously weighed collagen strip taken from the Achilles tendon of another rabbit. As the blood flowed over the strip, platelets and other cells adhered to it to form a thrombus until there was no further gain in weight of the strip. The blood was returned to the first rabbit under gravity. When eicosapentaenoic acid was infused into the blood passing over the loaded strip a decrease in weight was observed, showing that at least part of the aggregated platelets and other cells had been disaggregated from the loaded strip. This ability of eicosapentaenoic acid to bring about dispersion or disaggregation of thrombus is important in the treatment of thrombosis, and also in its prophylactic treatment. When a thrombus is being formed in an artery (or vein) there is a reduction in the blood flow (which flow would be completely stopped if the vessel were to become completely occluded). This reduction in blood flow brings about ischaemia, which produces pain. The reduced blood flow can, however, carry therapeutic materials to the site of thrombus formation. *In vivo* with eicosapentaenoic acid the residual blood flow and any blood flow in the collateral circulation can carry the acid to the site of thrombus formation where the eicosapentaenoic acid and its metabolites can disaggregate the thrombus and restore full blood flow. Accordingly, this invention also provides a method of restoring full blood flow in a partly occluded blood vessel by administering eicosapentaenoic acid. The administration can also be used prophylactically to help to keep blood vessels clear.

We have also found that human platelets, when pre-incubated with eicosapentaenoic acid and then incubated with arachidonic acid and stimulated with ADP, aggregate less readily than when

the pre-incubation is carried out with arachidonic acid. This suggested to us that, if human platelets could be 'primed' with eicosapentaenoic acid, they would be less susceptible to ADP stimulation and so less liable to form thrombi.

The Applicants believe, although they do not wish to be bound by this belief, that *in vivo* the eicosapentaenoic acid, in contrast to arachidonic acid, not only itself has an anti-aggregatory effect on blood platelets but its metabolites, presumably prostaglandins of the Δ-17 series, also have an anti-aggregatory effect on the platelets, or at worst a reversible aggregating effect, whereas many of the metabolites of arachidonic acid, such as PGH_2 and TXA_2, have an irreversible aggregating effect on platelets. This net anti-aggregating profile for eicosapentaenoic acid is, the Applicants believe, responsible for its surprisingly beneficial properties.

The dose of eicosapentaenoic acid needed for therapeutic or prophylactic effect will vary with the route of administration and the nature of the condition being treated, but will generally be at least 1 gram (g), preferably from 1.5 to 7.5 g, especially 2 to 6 g for example 5 g per day. This is the dose for an average 70 g man and the dose for other men or animals will generally vary pro-rata according to their weight, i.e. about 20 to 100 mg/kg.

Eicosapentaenoic acid may be added to extra-corporeally circulating blood to prevent, substantially or completely, aggregation of blood platelets induced by contact with the machine or with other non-tissue materials.

Eicosapentaenoic acid is known to be present in oysters and other sea foods, in cod liver oil and in other oils, e.g. menhaden oil, from which it may be extracted by methods known in the art or described in the literature. The eicosapentaenoic acid may also be synthesised by conventional methods of synthetic organic chemistry. The route chosen will depend on the availability of suitable starting materials, and on the relative costs of the various routes available to provide eicosapentaenoic acid of the right quality for human medical or veterinary use. Care should be taken in extractive and preparative processes to avoid, or keep low, the isomerisation of *cis*-double bonds to *trans*-double bonds.

The amounts of eicosapentaenoic acid in naturally occurring or readily extractable materials, such as cod liver oil or menhaden oil, are such that it would not be possible to obtain the desired amount of eicosapentaenoic acid by administering them without also administering too many calories in the form of other fatty acids. Furthermore, as cod liver oil (and other fish oils) is rich in vitamin A (at least 850 international units (I.U.) per gram) and vitamin D (at least 85 I.U. per gram) administering enough cod liver oil to give the necessary amount of eicosapentaenoic acid would administer amounts of these vitamins greatly exceeding the recommended daily dose for humans and would lead to hyper-vitaminosis. The recommended daily dose is 5000 I.U. for vitamin A and 400 I.U. for vitamin D in humans. In the U.S.A. the Food and Drugs Administration has laid down that the daily intake of vitamin A should not exceed 10,000 I.U. and of vitamin D should not exceed 400 I.U. Amounts above this require a doctor's prescription.

Therefore to avoid complications, which may arise through the recipient receiving vitamin doses for other medicinal reasons, or at his or her own instigation, a formulation is preferably provided which comprises eicosapentaenoic acid, or a pharmaceutically acceptable salt, ester or amide thereof, and a pharmaceutically acceptable carrier, the formulation being substantially free of vitamins.

Because of the complex and to some extent uncertain effects of acids less unsaturated than eicosapentaenoic acid a formulation is preferably provided comprising eicosapentaenoic acid, or a pharmaceutically acceptable salt, ester, or amide thereof, and a pharmaceutically acceptable carrier, the formulation being substantially free of other, less unsaturated acids, or their salts, esters or amides. In eicosapentaenoic acid obtained from natural sources, such as fish oils, there is usually a proportion of (all-Z) 7,10,13,16,19-docosapentaenoic acid (hereinafter referred to as docosapentaenoic acid) and/or of (all-Z)4,7,10,13,16,19-docosahexaenoic acid (hereinafter referred to as docosahexaenoic acid) (as such or as their derivatives i.e. their esters, salts or amides). It is not necessary to try to remove these equally or more unsaturated acids (or their derivatives), because they behave in a way similar to eicosapentaenoic acid, but are less active.

The excessive calorie intake mentioned above, if, for example, cod liver oil or menhaden oil were used as the source of the eicosapentaenoic acid, may be substantially overcome, although some control of calorie intake in the remainder of the diet may still be necessary, by administering a formulation comprising eicosapentaenoic acid, or a pharmaceutically acceptable salt, ester or amide thereof, and a pharmaceutically acceptable carrier, at least 50%, e.g. greater than 56%, by weight of the fatty acid content of the formulation being provided by eicosapentaenoic acid. However, if the eicosapentaenoic acid is to be administered without modification of the recipient's diet, the acid (and any docosapentaenoic acid or docosahexaenoic acid) should represent at least 90%, preferably at least 95% or all, by weight of the fatty acid content of the administered material.

Arachidonic acid should preferably be absent or at most should be no more than 5% of the fatty acid content. For example a preferred quality of eicosapentaenoic acid comprises at least

90% of the acid, about 2% of each of arachidonic and dihomo-γ-linolenic acids, the balance being docosahexaenoic, docosapentaenoic, palmitic or oleic acids, and other pharmaceutically acceptable fatty acids. If vitamins are present, as they may be, they should preferably not be present in amounts that would lead to their recommended daily intake being exceeded.

Formulations used according to the invention should also be free of saturated fatty acids and their salts, esters or amides. Preferably the formulations should be free of unsaponifiable materials.

By administering the eicosapentaenoic acid as at least 50%, preferably at least 90%, of the fatty acid content, it should be possible to avoid substantial alteration of the diet of the recipient, except perhaps to reduce slightly the calorific content of the diet to allow for the extra calories from the eicosapentaenoic acid (and other fatty acids). However, if preferred, it may be possible to administer the eicosapentaenoic acid by replacing, say, butter and/or ordinary margarine by a special margarine, e.g. of the emulsion type, formulated so that in normal usage the recipient would receive the required amount of the eicosapentaenoic acid. Cooking oils and fats may also be similarly formulated to contain the eicosapentaenoic acid.

The eicosapentaenoic acid (and other acids) need not be used as the acid itself but may be used as its pharmaceutically acceptable salts, esters or amides (which would be measured as their acid equivalents). Esters or amides which can be converted *in vivo* to the acid and other pharmaceutically acceptable products may be used, the preferred ester being the triglyceride or ethyl ester, but the methyl ester could perhaps also be used. The alcohol used to esterify the acid should preferably be non-polymeric and should preferably contain no more than three hydroxyl groups in the molecule. Further, the ester used is preferably not the cholesteryl ester as this would lead to some cholesterol being liberated which may lead to an increase in the serum cholesterol level. The preferred salts are the sodium or potassium salts or any other pharmaceutically acceptable solid salt, as these are more suitable for making into tablets. Tablets may comprise a pharmaceutically acceptable solid derivative, e.g. a salt, of eicosapentaenoic acid.

As eicosapentaenoic acid is highly unsaturated, it and its derivatives are readily oxidisable and formulations containing them should preferably also contain anti-oxidants, such as butylated hydroxy toluene, butylated hydroxy anisole, propyl gallate, a pharmaceutically acceptable quinone and α-tocopherol. Some anti-oxidants may also contribute to the anti-thrombo-embolic effect.

Although it is preferred to administer the eicosapentaenoic acid (or its salts, esters or amides) (active compound) orally as this is a convenient route for routine administration, the active compound may be administered by any route by which it may be successfully absorbed, e.g. parenterally (i.e. subcutaneously, intramuscularly or intravenously), rectally or, in the case of women, vaginally.

While it is possible for the active compound to be administered as a raw chemical or as a simple mixture of components, it is preferable to present it as a pharmaceutical formulation. The formulations, both for veterinary and for human medical use, of the present invention comprise the active compound as above defined, together with one or more acceptable carriers therefor and optionally other therapeutic ingredients. The carrier(s) must be 'acceptable' in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof. Formulations which contain eicosapentaenoic acid itself are preferably non-aqueous. Unit doses, e.g. tablets or capsules, of a formulation generally contain from 0.25 to 1.0 g, e.g. 0.5 g, of the active compound. Generally, three doses would be administered per day.

Formulations which may be used include those suitable for oral, rectal, vaginal, or parenteral (including subcutaneous, intramuscular and intravenous) administration.

As eicosapentaenoic acid itself is a liquid and tends to be unpalatable, it is preferably administered per orally in a capsule, for example one of soft gelatin, so that the eicosapentaenoic acid is not tasted. The capsule would generally be of a size to permit the required dose of eicosapentaenoic acid to be administrable in one, two or three capsules at each dose taking and so a capsule would be generally about 0.5 ml in size. Another way of disguising the taste of the acid is to formulate it as an emulsion to be taken orally. The acid could also be formulated to be spontaneously emulsifiable on being taken orally or on being diluted before administration. An emulsion could also be of the multiple type; e.g. the acid could be made into an oil-in-water emulsion with a pharmaceutically acceptable surface active agent and then this emulsion could be emulsified in another oil, e.g. arachis oil. Alternatively, the acid could be similarly formulated into a water-in-oil emulsion and then this emulsion itself emulsified in water. The various types of emulsion could be presented as an oral gel or as a stiff emulsion, such as an emulsion margarine. Other methods of disguising the taste are to absorb the acid onto a carrier or carriers such as kaolin, chalk, calcium phosphate, calcium sulphate, starch, a micro-crystalline cellulose, or methyl or other modified cellulose. The resulting powder could be sold as such or flavoured, and perhaps made into tablets or capsules, each tablet or capsule containing, for example, about 0.5 g of eicosapentaenoic acid as such or in the form of a solid derivative. Tablets could be film-

or sugar-coated.

As for the salts, e.g. the sodium or potassium salts, these also tend to be unpalatable and tablets containing them, and representing for example 0.5 g of acid, should preferably be coated e.g. by film or sugar. Other methods of oral administration, e.g. cachet or lozenge, may also be used in appropriate circumstances. The esters or amides may be formulated as for the acid or the salts, depending on whether they are liquid or solid, respectively.

If desired an oral formulation can be presented as a sustained release formulation, for example as beads or micro-capsules in a capsule.

A formulation for intramuscular administration could be in the form of an emulsion. A formulation for intravenous injection could be in the form of a mixture that would spontaneously emulsify upon injection.

For rectal administration the acid or derivative could be formulated into a suppository in a triglyceride base, e.g. cocoa butter, a Witepsol or Suppocire or placed in a soft gelatin suppository capsule.

The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. All methods include the step of bringing into association the active compound with the carrier which constitutes one or more accessory ingredients. In general the formulations are prepared by uniformly and intimately bringing into association the active compound with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product into the desired formulation. In the present specification and claims the term "carrier" includes one which is suitable for administration to a recipient and substantially encloses the active compound e.g. the body of a capsule or the coating on a coated tablet.

To improve the effectiveness of the eicosapentaenoic acid, the formulation may also include a phosphodiesterase inhibitor, such as theophylline or dipyridamole.

Accordingly, the present invention provides:—

(a) (all-Z)-5,8,11,14,17-eicosapentaenoic acid, or a pharmaceutically acceptable salt, ester or amide thereof, for use in the treatment or prophylaxis of a thrombo-embolic condition;

(b) a formulation comprising (all-Z)-5,8,11,14,17-eicosapentaenoic acid or a pharmaceutically acceptable salt, ester or amide thereof, and a pharmaceutically acceptable carrier, at least 50% of the fatty acid content of the formulation being provided by (all-Z)-5,8,11,14,17-eicosapentaenoic acid (i.e. as such or as a derivative);

(c) a formulation comprising (all -Z)-5,8,11,14,17-eicosapentaenoic acid, or a pharmaceutically acceptable salt, ester or amide thereof, and a pharmaceutically acceptable carrier, the formulation being substantially free of vitamins;

(d) a formulation comprising (all -Z)-5,8,11,14,17-eicosapentaenoic acid, or a pharmaceutically acceptable salt, ester or amide thereof, and a pharmaceutically acceptable carrier, the formulation being substantially free of other, less unsaturated acids (i.e. as such or as their derivatives);

(e) a method of preparing a pharmaceutical formulation according to (b), (c) or (d);

(f) a margarine, butter, cooking oil or fat formulation including (all -Z)-5,8,11,14,17-eicosapentaenoic acid or a salt, ester or amide thereof in an amount to provide at least 3% by weight of the eicosapentaenoic acid (i.e. as such or as a derivative);

(g) a method for the treatment or prophylaxis of a thrombo-embolic condition in a mammal including man, which comprises administering a therapeutic or prophylactic anti-thrombo-embolic amount of (all -Z)-5,8,11,14,17-eicosapentaenoic acid or a pharmaceutically acceptable salt, ester or amide thereof;

(h) a method according to (g) using a formulation according to any one of (b), (c) and (d).

(i) a method of increasing the bleeding time of a mammal, including man, which comprises administering an effective amount of (all-Z)-5,8,11,14,17-eicosapentaenoic acid, or a salt, ester or amide thereof;

(j) a method of dispersing or disintegrating an already formed thrombus or platelet clump in a mammal including man, which comprises administration of an effective amount of (all-Z)-5,8,11,14,17-eicosapentaenoic acid or a salt, ester or amide thereof;

(k) a method of modifying and/or controlling adherence of blood platelets to damaged tissue in a mammal, including man, which comprises administering an effective amount of (all-Z)-5,8,11,14,17-eicosapentaenoic acid or a salt, ester or amide thereof;

(l) a method of rendering blood platelets in a mammal, including man, less readily aggregatable and/or more readily disaggregatable from one another by administering an effective amount of (all-Z)-5,8,11,14,17-eicosapentaenoic acid or a salt, ester or amide thereof;

(m) a method according to any one of methods (i) to (l) using a formulation according to any one of (b), (c) and (d);

(n) a method of extra-corporeally circulating blood to and from a mammal, including man, which includes administering to the blood, intra- or extra-corporeally, an amount effective to prevent, substantially or completely aggregation of blood platelets, of (all-Z)-5,8,11,14,17-eicosapentaenoic acid or a salt, ester or amide thereof; and

(o) a method of restoring or maintaining full blood flow in a partly occluded blood vessel in a mammal, including man, which comprises administering eicosapentaenoic acid or a salt, ester, or amide thereof.

The present invention is illustrated by the following Examples.

EXAMPLE 1

Blood from human volunteers who had not taken aspirin for the previous two weeks was collected from an ante-cubital vein in sodium citrate (0.11 M), 1 part of citrate to 9 parts of blood. Plasma was separated from the blood by centrifugation at 160 g (5 minutes) as a platelet rich plasma (PRP).

Studies on platelets were performed with arachidonic acid (AA), eicosapentaenoic acid (EPA) prepared as potassium salts (see Schrör, K., Moncada, S., Ubatuba, F.B., and Vane, J.R., Eur. J. Pharmac., 1978, *47*, 103) and with ADP or thrombin in a coagulation apparatus e.g. 'Fibromate' (Bie & Bernsted Copenhagen, Denmark).

Aggregation was recorded both turbidimetrically and nephelometrically in a cylindrical cuvette, containing 300 μl of PRP at 37°C and stirred magnetically at 800 rpm; alternatively a Payton dual channel aggregometer was used with 500 μl PRP.

In contrast to AA, EPA did not induce aggregation in human PRP at concentrations of EPA (1.33, 2.66 and 5.3 mM) about 4 or more times greater than AA (0.33, 0.66 and 1.3 mM). At lower concentrations in the range of from 0.01 to 0.5 mM EPA somewhat inhibited platelet aggregation induced by ADP (2 μM) in the human PRP.

The anti-aggregating effect of EPA (0.065 mM), however, was not due to its conversion by platelet cyclo-oxygenase because the anti-aggregating effect was present with aspirin-treated platelets which did not respond to AA (0.065 mM) but were aggregated by thrombin (0.04–0.4 U/ml), and the anti-aggregating effect was also present in first phase aggregation induced by ADP (2 to 5 μM) in aspirin-treated platelets.

EXAMPLE 2

Vascular tissue (thoracic and abdominal aorta) was obtained from freshly killed rats. Approximately 100 mg of tissue was chopped and washed once in ice cold Tris buffer (0.05 M, pH 7.5). After testing its ability to inhibit thrombin-induced platelet aggregation when added to the platelet cuvette, the tissue was washed several times in 10 ml of ice cold Tris buffer to remove blood and adhering platelets. The tissue was then quickly frozen to -60°C, crushed to a coarse powder and resuspended in five volumes of Tris buffer. This suspension of vascular tissue was kept on ice during the experiments and used for incubation studies.

Blood was obtained from the ante-cubital vein of human volunteers that had taken aspirin (1.5 g per day) for the 3 days before blood sampling. Washed human platelets were obtained from this blood as described by Vargaftig, B.B., Tranier, Y., and Chignard, M., (Prostaglandins, 1974, *8*, 133). Aggregation tests were carried out as in Example 1.

To see if the suspension of vascular tissue could synthesise any material having an anti-aggregatory effect on human platelets, platelets were obtained as described above from volunteers who had taken aspirin, so that their platelets could not produce prostaglandin endoperoxides that could be utilised by the vascular tissue to make anti-aggregatory material. Moreover, washed platelets were used to avoid any possibility of the vascular tissue utilizing any AA in the plasma. Under these conditions anti-aggregating activity could be formed by the vascular tissue only from endogenous or exogenously added precursors.

The initial suspension of vascular tissue (10 to 50 μl) described above inhibited aggregation induced by thrombin (0.04 to 0.4 U/ml). This inhibitory activity was abolished by repeated washing (5 to 20 times) of the tissue by centrifuging (30 seconds in an Eppendorff centrifuge), pouring off the supernatant and resuspending in fresh buffer (0.5 ml). The general level of inhibitory activity against primary phase aggregation induced by ADP (2 to 5 μM) or aggregation induced by thrombin (0.04 to 0.4 U/ml) could be restored by adding washed vascular tissue and EPA to the washed platelets from aspirin-treated volunteers. The generation of anti-aggregating activity was prevented by the pretreatment of the washed vascular tissue with indomethacin (5 to 10 μg/ml). Thus, the vessel wall cyclo-oxygenase could utilise EPA to form anti-aggregating activity.

The anti-aggregating activity formed might have been due to displacement of endogenous AA by EPA and not to direct utilization of EPA. However, the same concentrations of DHLA incubated with washed vascular tissue did not lead to the formation of anti-aggregating material and so DHLA does not displace AA.

EXAMPLE 3

The effect of Eicosapentaenoic Acid on Bleeding Time In the Rabbit

Four male New Zealand white rabbits (Ranch) weighing 2.0 to 2.5 kg were anaesthetized with sodium pentobarbitone (40 mg/kg). The marginal ear vein was cannulated for infusions (0.1

ml/min) of eicosapentaenoic acid. The potassium salt of eicosapentaenoic acid (95% pure and containing about 2% AA and 2% DHLA, balance C-18 fatty acids) was dissolved in 50 mM Tris-HCl buffer pH 8.0 kept on ice and shielded from light. Infusions of either the Tris vehicle or eicosapentaenoic acid were made 5 minutes before and continuously during the measurement of bleeding time.

The internal surface of the ear without the cannula was carefully shaved. The ear was transilluminated so that blood vessels were clearly visible. Cuts, approximately 0.4 cm long and deep enough to cause an updwelling of blood within 15 seconds, were made with a new scalpel blade in an area free of visible blood vessels and in a direction parallel to the nearest blood vessel. The cut was gently blotted every 15 seconds with filter paper (Whatman No. 1).

Bleeding time was measured to the nearest 15 seconds from the time of incision until dots of blood were no longer visible on the filter paper. If there was a plasma exudate from the cut, the end point was considered as the time when the exudate no longer had a reddish tinge. When bleeding time was longer than 10 minutes, the cut was then blotted every 30 seconds. The bleeding time at each dose was a mean of 3 estimations.

Two rabbits were pretreated with aspirin 100 mg/kg i.v. injection 4 hours before the experiment. Two rabbits were given 0.5 ml Tris pH 7.5 in 4 ml saline in the same way to act as controls. The results obtained are set out in Tables 1 and 2.

Table 1
Controls i.e. no aspirin

EPA Dose µg/kg/min	Bleeding Time* minutes	
	Rabbit 1	Rabbit 2
0	3.5	3.0
50		16.0
100	19.8	16.5
200		**23.0

Table 2
Pretreated with aspirin 100 mg/kg i.v. injection 4 hours before test begun

EPA Dose µg/kg/min	Bleeding Time* minutes	
	Rabbit 3	Rabbit 4
0	5.3	4.7
100	7.3	6.3
200	4.5	**7.5

* Mean of 3 estimations
** Rate of infusion 0.2 ml/min

Accordingly when treated with aspirin, the rabbits showed little or no increase in bleeding time.

A rabbit treated with 75% prue EPA gave similar results after allowing from the lower purity of the acid.

EXAMPLE 4
Conversion of Eicosapentaenoic Acid in the Circulation of the Dog

Intravenous infusion of eicosapentaenoic acid (0.2 to 2 mg $kg^{-1}min^{-1}$) caused systemic and pulmonary hypotension in chloralose anaesthetized dogs. Blood-bathed isolated strips of bovine coronary artery and rabbit coeliac artery are known to be relaxed by the powerfully anti-aggregatory material PGI_2 (5 to 10 ng/ml). When treated with antagonists of catecholamines and angiotensin II, these bioassay tissues, bathed in arterial blood relaxed during infusion of eicosapentaenoic acid (0.6 to 2 mg $kg^{-1}min^{-1}$, 2 dogs), by an amount equivalent to about 10 to 20 ng/ml PGI_2 at the highest rates. In one of these dogs after administration of indomethacin

(5 mg/kg), subsequent infusion of eicosapentaenoic acid (2 mg $kg^{-1}min^{-1}$ for 10 min) still caused hypotension but did not release any detectable activity in the bioassay tissues.

EXAMPLE 5

Disaggregating effect of Eicosapentaenoic Acid in the Rabbit

Rabbits (2–3 kg) were anaesthetized with pentobarbitone sodium 30 mg/kg and heparinized (2000 U/kg). A carotid artery was dissected and blood was exteriorized and delivered with a roller pump to superfuse a strip of collagen from the Achilles tendon of a different rabbit. As the blood flowed over the tendon strip, the strip increased in weight over a period of 35 min up to a maximum of from 180 to 200 mg. Thereafter any decrease in weight was due to platelet disaggregation.

Eicosapentaenoic acid infused intravenously (50–500 μg/kg/min) into five rabbits induced small disaggregating effects (approximately 20 mg). This effect of eicosapentaenoic acid could be inhibited by pre-treating the rabbits with aspirin (150 mg/kg).

EXAMPLE 6

A soft gelatin capsule to contain about 0.5 ml was sterilised and then filled with a composition containing more than 90% of EPA, about 2% AA, about 2% DHLA with the balance including palmitic and oleic acids. The capsule was then sealed.

The capsule used may be transparent or coloured, and may also be of the hard gelatin type or made of polymethyl methacrylate for example.

EXAMPLE 7

A tablet formulation comprised:—

Sodium eicosapentaenoate	281 mg
Starch	62 mg
Lactose	250 mg
Polyvinyl pyrrolidone	3.5 mg
Magnesium Stearate	3.5 mg
Butylated hydroxy toluene	2 ppm
TOTAL	600 mg

The tablet was coated with sugar, although other coating agents could be used.

EXAMPLE 8

The formulation described in Example 7 in untabletted powder form may be used to fill hard gelatin capsules with 600 mg of the formulation.

EXAMPLE 9

About 250 g of a conventional soft margarine formulation was thoroughly mixed with 8 g of eicosapentaenoic acid until a smooth consistency was reached.

EXAMPLE 10

Male New Zealand rabbits (2–2.5 kg) were given aspirin (10 or 100 mg/kg i.v.). A control group received only the liquid vehicle used for dissolving the aspirin. Two to four hours later the animals were anaesthetised with pentobarbitone and cutaneous bleeding time was measured as described in Example 3, before and during the infusion of EPA (potassium salt, 95% pure as used in Example 3) at different rates (1,50,200 or 400 μg/kg/min). Duplicate or triplicate measurements were done for each condition. The results obtained are set out in Table 3 below. Aspirin (10 mg/kg produced a small but significant ($p<0.0001$) increase in bleeding time, the average of triplicate determination in five rabbits was 489 ± 27 s (mean ± s.e.m.) compared to the controls 278 ± 48 s. The value of 288 ± 11 s in the animals treated with a large dose (100 mg/kg) of aspirin was not significantly higher than the control.

In the group receiving no aspirin, EPA (1 μg/kg/min) prolonged bleeding time by more than 100% ($p<0.00001$). A further increase in bleeding time is observed at higher rates of infusion and a plateau value of about 1000 sec is attained at the rate of 50 μg/kg/min.

In the animals treated with aspirin (10 or 100 mg/kg) EPA failed to produce a significant modification in the bleeding time.

Table 3

Pretreatment	EPA μg/kg/min 0	1	50	200	400
None	278 ± 48 n = 8	642 ± 69 n = 5	861 ± 69 n = 5	961 ± 87 n = 4	1020 ± 89 n = 4
ASA 10 mg/kg	489 ± 27 n = 5	607 ± 97 n = 5	678 ± 115 n = 5	839 ± 160 n = 5	693 ± 114 n = 4
ASA 100 mg/kg	288 ± 11 n = 5	309 ± 13 n = 3	327 ± 82 n = 3	428 ± 86 n = 4	364 ± 26 n = 3

Mean bleeding time in seconds ± s.e.m.
ASA = Aspirin

EXAMPLE 11

Using the method of Example 3, the effects were observed of various fatty acids on the aggregation induced by 11α, 9α-epoxymethano-15-hydroxyprosta-5,13-dienoic acid (an analogue of PGH_2) (Upjohn) of aspirin-treated platelets in human PRP.

In each test the fatty acid was incubated with the platelets for about 6 minutes before the PGH_2 analogue was added; the amount of fatty acid used was about 1.5 mM. The amounts of inhibition obtained six minutes after addition of PGH_2 analogue are set out in Table 4.

Table 4

Acid	% Inhibition
Eicosapentaenoic acid	100
(all-Z)-9,12,15-octadecatrienoic acid	40
(all-Z)-9,12-octadecadienoic acid	55
(all-Z)-6,9,12-octadecatrienoic acid	63
Z-9-octadecaenoic acid	68
Control	0

Eicosapentaenoic acid also exhibited 100% inhibition at a concentration of 1.0 mM and about 95% inhibition at a concentration of 0.5 mM.

It should be noted that (all-Z)-9,12,15-octadecatrienoic acid and (all-Z)-6,9,12-octadecatrienoic acid, which are 2,3-dinor analogues of the 11,14,17-eicosatrienoic acid and 8,11,14-eicosatrienoic acid used in the paper by Silver *et al* referred to above, were not very effective as anti-aggregation agents as compared to eicosapentaenoic acid.

CLAIMS

1. (All-Z)-5,8,11,14,17-eicosapentaenoic acid, or a pharmaceutically acceptable salt, ester, or amide thereof as an active agent for the treatment of a thrombo-embolic disorder or disease in a mammal.

2. A formulation as an active agent for the treatment of a thrombo-embolic disorder or disease in a mammal, and comprising (all-Z)-5,8,11,14,17-eicosapentaenoic acid, or a pharmaceutically acceptable salt, ester or amide thereof, and a pharmaceutically acceptable carrier; at least 50% by weight of the fatty acid content of the formulation being provided by (all-Z)-5,8,11,14,17-eicosapentaenoic acid or the derivative thereof.

3. A formulation according to claim 2 in which at least 90% by weight of the fatty acid content of the formulation is provided by (all-Z)-5,8,11,14,17-eicosapentaenoic acid or a derivative thereof.

4. A formulation according to claim 2 or 3 which includes in its fatty acid content about 2% of arachidonic acid, about 2% of dihomo-γ-linolenic acid, with the balance being pharmaceutically acceptable and comprising docosahexaenoic, docosapentaenoic, palmitic, oleic acid or other fatty acids, or derivatives thereof.

5. A formulation as an active agent for the treatment of a thrombo-embolic disorder or disease in a mammal and comprising (all-Z)-5,8,11,14,17-eicosapentaenoic acid, or a pharmaceutically acceptable salt, ester or amide thereof, and a pharmaceutically acceptable carrier, the

formulation being substantially free of other less unsaturated acids or derivatives thereof.

6. A formulation according to claim 2 or 3 substantially free of other, less unsaturated acids or derivatives thereof.

7. A formulation as an active agent for the treatment of a thrombo-embolic disorder or disease in a mammal and comprising (all-Z)-5,8,11,14,17-eicosapentaenoic acid, or a pharmaceutically acceptable salt, ester or amide thereof, and a pharmaceutically acceptable carrier, the formulation being substantially free of vitamins.

8. A formulation according to any one of claims 2 to 6 substantially free of vitamins.

9. A formulation according to any one of claims 2 to 8 in which the eicosapentaenoic acid is present in the form of its sodium or potassium salt.

10. A formulation according to any one of claims 2 to 8 in which the eicosapentaenoic acid is present as its triglyceride or ethyl ester.

11. A formulation according to any one of claims 2 to 8 in which eicosapentaenoic acid itself is used.

12. A formulation according to any one of claims 2 to 11 including an antioxidant.

13. A formulation according to any one of claims 2 to 12 including a flavouring agent.

14. A formulation according to any one of claims 2 to 13 in which the carrier is or includes a capsule enclosing the remainder of the formulation.

15. A formulation according to any one of claims 2 to 13 in which the eicosapentaenoic acid, salt, ester or amide forms a disperse phase in the carrier which is a liquid.

16. A formulation according to claim 15 in a capsule.

17. A formulation according to any one of claims 2 to 13 in tablet form, the carrier being solid.

18. A formulation according to any one of claims 2 to 17 in unit dosage form.

19. A formulation according to claim 18 containing 0.25 to 1.0 g of eicosapentaenoic acid, as such or as a salt, ester or amide.

20. A formulation according to any one of claims 2 to 19 substantially free of saturated fatty acids or their salts, esters or amides.

21. A tablet as an active agent for the treatment of a thrombo-embolic disorder or disease in a mammal and comprising a solid derivative of (all-Z)-5,8,11,14,17-eicosapentaenoic acid.

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